Pattern of Graft- and Host-Specific MHC Class II Expression in Long-Term Murine Cardiac Allografts

Origin of Inflammatory and Vascular Wall Cells

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In solid-tissue allografts, donor vascular cells as well as recipient inflammatory cells can express MHC class II molecules. However, it is uncertain how much residual donor endothelium persists and to what extent donor versus recipient MHC class II expression can contribute to the ongoing immune response, especially in long-term grafts. To establish the origin of class-II-expressing cells in the allograft, we evaluated the expression of donor- or recipient-specific MHC class II molecules in murine cardiac allografts. Donor hearts from BALB/c (H-2^d) mice were transplanted into C57BL/6 (B6, H-2^b) recipients; B6 isografts served as controls. Untreated allografts ceased functioning at approximately 7 days with severe parenchymal rejection. Allografts from recipients treated with anti-CD4 and anti-CD8 MAb after transplantation were explanted at 8 to 12 weeks and demonstrated intimal fibroproliferative lesions with a mild parenchymal mononuclear cell infiltrate. Class II expression in isografts was limited to epicardial macrophages. Both acutely rejecting and long-term allografts contained abundant macrophages expressing recipient class II molecules. Occasional cells (passenger leukocytes) in untreated, acutely rejecting allografts bore donor class II molecules; long-term allografts contained few such cells. In contrast, vascular endothelial and medial smooth muscle cells consistently expressed donor class II molecules. These results suggest that ongoing MHC class II expression in donor vascular cells, as well as in recipient macrophages, may contribute to sustained activation of host T cells with consequent release of cytokines that ultimately promote the development of graft arteriosclerosis. (*Am J Pathol* 1998, 153:69–79)

In 1989, we hypothesized that an allogeneic response stimulated by foreign MHC class II molecules promotes the development of graft vascular disease.¹ We subsequently showed that the endothelium of vessels within transplanted human hearts indeed bears MHC class II molecules and that sustained allogeneic class II expression on the graft endothelium may contribute to the development of graft vascular disease.² These studies used reagents that recognize framework determinants on the MHC class II molecules rather than discrete polymorphic regions. Therefore, it remained uncertain to what extent donor vascular wall cells capable of instigating an immune response might persist in long-term allografts. Conversely, it was unknown how extensively host-derived vascular cells might insinuate into grafts, in which case host immune recognition would no longer occur. Moreover, the persistence of donor immunogenic passenger leukocytes in long-term allografts remains controversial.

For these reasons, defining the origin of these cells in long-term allografts of solid organs has considerable importance. There is older evidence from a rat experimental model that the endothelium of long-term aortic allografts contains some recipient-derived cells.³ In contrast, recent reports using immunohistochemical staining for donor-specific MHC class II molecules have demonstrated that endothelial cells (ECs) lining vessels of acutely rejecting allografts in rodents are of donor origin.^{4–7} Nevertheless, it is unclear whether the ECs that line vessels of longer-term allografts originate from the donor and/or recipient.

The availability of mouse strains of well defined histocompatibility haplotype, as well as allospecific antisera capable of selectively differentiating MHC molecules, should permit unambiguous determination of the animal of origin of all cell types. We have therefore used long-term total allogeneic-mismatched murine cardiac allo-

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Table 1. Strain Combinations, Treatment, and Morphology

Combination	Treatment	n	Duration	PR	GAD
Isograft (B6 to B6)	None	4	12 weeks	0 ± 0	0 ± 0
Isograft (B6 to B6)	Anti-CD4 MAb Anti-CD8 MAb	4	12 weeks	0 ± 0	0 ± 0
Allograft (B/c to B6)	None	6	7.0 ± 0.2 days*	2.8 ± 0.4	0.3 ± 0.1
Allograft (B/c to B6)	Anti-CD4 MAb Anti-CD8 MAb	6	8–12 weeks	0.8 ± 0.3	1.5 ± 0.3

Values represent the mean ± SD. PR, parenchymal rejection; GAD, graft arterial diseases.

*Explanted at time of graft failure.

grafts to examine the pattern of donor *versus* recipient MHC class II expression in allograft ECs and vascular smooth muscle cells (SMCs) as well as allograft mononuclear inflammatory cells.

Materials and Methods

Animals

Male BALB/c (B/c, H-2^d) and C57BL/6 (B6, H-2^b) mice were obtained from Taconic Farm (Germantown, NY) and were used at 10 to 12 weeks of age; body weight was approximately 25 g. B/c mice were used as allograft donors and B6 mice were used as recipients. These strains are disparate in major histocompatibility complex (MHC) class I, MHC class II, and multiple non-MHC alloantigens. B6 isografts were used as controls.

The mice were maintained in the Harvard Medical School animal facilities on acidified water. Sentinel animals surveyed serologically for viral pathogens were negative in the room in which these mice were housed. All experiments conformed to animal care protocols approved by the institutional review group.

Heterotopic Cardiac Transplantation

Heterotopic cardiac transplantation was performed using a modification of the method described by Corry et al.⁸ In brief, donors and recipients were anesthetized with Metofan (Pittman-Moore, Mundelein, IL). Donor hearts were perfused with chilled and heparinized saline via the inferior vena cava and harvested after ligation of the vena cava and pulmonary veins. The aorta and pulmonary artery of donor hearts were anastomosed to the abdominal aorta and inferior vena cava, respectively, of recipients using microsurgical techniques. Ischemic time was routinely approximately 25 minutes, with a success (long-term survival) rate of approximately 90%. The viability of the cardiac allograft was assessed by daily abdominal palpation. Cessation of the graft heartbeat was defined as the day of graft failure, typically indicating severe acute rejection as determined by histopathological examination.

Immunosuppressive Treatment and Experimental Groups

Table 1 shows the various experimental groups. Immunosuppression was attained by weekly intraperitoneal

injections of 0.2 ml of ascites or comparably concentrated antibody preparations containing anti-CD4 (GK1.5) and anti-CD8 (2.43) monoclonal antibodies (MAbs) beginning 4 days after transplantation.⁹ This protocol permits a single 4-day early acute rejection episode, followed by complete CD4 and CD8 cell ablation and therefore long-term suppression of any further immune-specific response. The model permits long-term (at least 12 weeks) survival of complete allogeneic-mismatched allografts (not possible in our hands with other protocols thus far published) and yields graft arteriosclerosis lesions histologically identical to those seen with conventional pretransplant MAb treatment.^{9,10}

Monoclonal Antibodies

GK1.5 (anti-CD4) and 2.43 (anti-CD8) MAbs for immunosuppression were prepared from hybridoma clones (American Type Culture Collection, Rockville, MD) and used as ascites preparations or from threefold concentrations of serum-free supernatants from an artificial capillary system (Cellmax, Celluco, Rockville, MD); antibodies for CD45, B220, Mac-3, and CD31 (PECAM-1) as well as biotinylated, fluorescein isothiocyanate (FITC)-conjugated or nonconjugated isotype- and species-specific secondary antibodies were purchased from PharMingen (San Diego, CA). Mouse monoclonal anti-smooth muscle α -actin antibody (1A4) was obtained from Sigma Chemical Co. (St. Louis, MO). Horseradish-peroxidase-conjugated anti-FITC antibodies were obtained from Boehringer Mannheim (Indianapolis, IN).

Monoclonal Antibodies for MHC Class II Immunostaining

Biotinylated, FITC-conjugated and nonconjugated MHC class II haplotype-specific MAbs as well as isotype-matched biotinylated, FITC-conjugated, and nonconjugated IgG_{2a} and IgG_{2b} control MAbs were purchased from PharMingen. The anti-I-A^b antibody was clone AF6–120.1, specific for the A_α^b chain, (IgG_{2a} isotype, κ light chain); according to the supplier, this antibody is cross-reactive with cells from H-2^k and H-2^u mice and weakly reactive with cells from H-2^p haplotype mice but not with cells from H-2^d animals. The anti-I-A^d antibody was clone AMS-32.1 (IgG_{2b} isotype, κ light chain); according to the supplier, this antibody is cross-reactive with cells from H-2^f, H-2^j, and H-2^v mice and weakly reactive with cells

Figure 1. Demonstration of the specificity of the anti-MHC class II antibodies used for immunohistochemical staining. Frozen sections of normal spleen from either C57BL/6 (H2^b, B6) or BALB/c (H2^d, B/c) were incubated with MAbs specific for I-A^b or I-A^d as described in Materials and Methods. No cross-reactivity of the MAbs was seen.

from H-2^k and H-2^q haplotype mice but not with cells from H-2^b animals. To verify directly lack of cross-reactivity under our immunostaining conditions, spleens from B6 and B/c mice were stained with both I-A-specific MAbs; as shown in Figure 1, each antibody specifically stains cells from only the appropriate MHC II haplotype animals.

Histological Techniques

Grafts were explanted either at the time of cessation of heartbeat or at 8 or 12 weeks and were transversely sectioned. One-half of each heart was fixed in 10% buffered formalin for routine morphological examination; paraffin-embedded sections of the fixed tissue were stained with hematoxylin and eosin (H&E) or with an elastic fiber stain using Weigert's method. The other half of each heart was frozen in OCT compound (Ames Co., Division of Miles Laboratories, Elkhart, IN) and stored at -80°C . Six-micron-thick sections were fixed in cold acetone for 2 minutes, incubated at room temperature with 5% normal goat serum in PBS for 20 minutes to block nonspecific binding sites, and subsequently immunostained with rat MAbs for CD45, CD4, CD8, Mac-3, or CD31 followed by biotinylated goat anti-rat IgG using an avidin-horseradish peroxidase-biotin complex method¹¹ (Vector Laboratories, Burlingame, CA) and an aminoethyl carbazole substrate (Vector Laboratories); hematoxylin was used for counterstaining. Controls included comparable concen-

trations of nonspecific rat IgG in the first step. For immunohistochemical staining for smooth muscle α -actin, frozen sections were stained with mouse 1A4 antibody (IgG_{2a} isotype) and then stained using biotinylated goat anti-mouse IgG_{2a} antibody. Controls included comparable dilutions of nonspecific mouse IgG_{2a} antibody in the first step.

Immunohistochemical staining for MHC II was performed using directly biotin-conjugated antibodies to MHC II haplotypes I-A^b or I-A^d and the avidin-horseradish-peroxidase-biotin complex method.¹¹ Controls included biotin-conjugated isotype-matched non-specific antibody in the first step.

Double-staining for MHC II and the EC marker CD31 was performed by sequential incubations with anti-CD31, biotin-conjugated anti-rat IgG, and avidin-alkaline-phosphatase-biotin complex method (Vector Laboratories) using the Vector Blue substrate kit (Vector Laboratories) and levamisole to block endogenous phosphatase activity. This treatment was followed by successive application of FITC-conjugated antibodies to MHC II haplotypes I-A^b or I-A^d and horseradish-peroxidase-conjugated anti-FITC; the slides were developed with the aminoethyl carbazole substrate. By this method, ECs stain blue, and MHC II expression is reflected by a red-brown stain; double-stained cells have a deep purple color. Controls included use of appropriately conjugated, isotype-matched irrelevant antibodies in the first step.

Table 2. Grading Criteria for MHC Class II Expression

Grade	Inflammatory cells	Vessel
0	None	No staining
1	Rare positive cells (<1%)	Focal and weak
2	Focal (1–10%)	Focal and moderate
3	Multifocal (10–50%)	Uniform and weak-moderate
4	Diffuse (>50%)	Uniform and intense

For immunofluorescent labeling of MHC class II molecules, frozen sections were incubated with biotin-conjugated antibodies to MHC II haplotypes I-A^b or I-A^d, followed by streptavidin-conjugated Texas Red (Amersham Life Science, Cleveland, OH), using standard techniques. Controls included comparable concentrations of isotype-matched, biotin-conjugated nonspecific antibodies in the first step.

Histological Evaluation

Grafts were analyzed by standard H&E and elastin stains, and the severity of parenchymal rejection *versus* graft arterial disease (GAD) was scored. Parenchymal rejection was graded using a scale modified from the International Society for Heart and Lung Transplantation¹² (0, no mononuclear cell infiltrate; 1, mild interstitial or perivascular infiltrate without necrosis; 2, focal infiltrates with necrosis; 3, multifocal infiltrates with necrosis; 4, widespread infiltrates with hemorrhage and/or vasculitis), and a GAD score was calculated from the number and severity of involved vessels (0, <10% vascular occlusion; 1, 10 to 25% occlusion; 2, 25 to 50% occlusion; 3, 50 to 75% occlusion; 4, >75% occlusion).¹⁰ All results are expressed as the mean $\pm$ SD.

Numerical grades for the intensity and extent of staining for MHC class II expression in both parenchymal inflammatory cells and vessels were averaged from scores determined by three independent, blinded observers (Table 2).

Results

Morphology of Parenchymal Injury and Vascular Lesions

All isografts, whether recipients were given anti-CD4 and anti-CD8 MAbs or not, continued to beat until harvest at 12 weeks and showed neither mononuclear cell infiltrates

nor graft vascular lesions (Table 1). Allografts in nonimmunosuppressed animals ceased functioning at approximately 7 days after transplantation and showed severe parenchymal rejection (Table 1; Figure 2A). Long-term allografts (8 to 12 weeks) in recipients immunosuppressed with weekly anti-CD4 and anti-CD8 MAbs beginning 4 days after transplantation exhibited an ongoing mononuclear cell infiltrate composed predominantly of macrophages.⁹ Arteries in these allografts exhibited intimal fibroproliferative vascular lesions (Table 1; Figure 2B), resembling lesions seen in other murine models of graft arteriosclerosis, as well as typical human graft arteriosclerosis.^{2,9,10}

Donor- or Recipient-Specific MHC Class II Expression in Transplanted Hearts

Freshly explanted native B6 hearts showed no constitutive class II expression in parenchymal or vascular wall cells (Figure 3A). Class II expression in isografts was confined to infiltrating macrophages in the epicardium, and no vascular or myocardial cells showed staining for class II (Table 3 and Figure 3, B and C). The results were the same regardless of whether isografted animals received weekly anti-CD4 and anti-CD8 MAbs (Figure 3, B and C) or not (not shown).

Allografts in nonimmunosuppressed recipients failing at approximately 7 days displayed abundant mononuclear inflammatory infiltrates expressing I-A^b (recipient-specific) molecules. These mononuclear I-A^b-positive cells co-localized with cells stained for CD45 and Mac-3 and therefore largely represent activated macrophages (Table 3 and Figure 4, A, B, E, and G) although scattered diffuse CD4- and CD8-positive T cells were also present in the allografts (not shown). In contrast, ECs (CD31-positive) and SMCs (smooth muscle α -actin-positive) in the vessels showed intense I-A^d (donor-specific) staining (Table 3 and Figure 4, C, D, and F). Occasional inflammatory cells around vessels also expressed I-A^d and are interpreted to represent passenger leukocytes (Figure 4F).

Allografts in immunosuppressed animals 8 weeks (Figure 5) and 12 weeks (Figure 6) after transplantation demonstrated I-A^b (recipient-specific) molecules on infiltrating macrophages (Mac-3 positive) in the expanded intima and around vessels, but not on ECs or medial SMCs (Table 3; Figures 5 and 6). In contrast, I-A^d (donor-specific) molecules were expressed in the vessel wall cells, including ECs and medial SMCs, but not in the

Table 3. Summary of Immunohistochemical Staining for Donor- or Recipient-specific MHC II

	MHC Class II			
	I-A ^b (B6-specific)		I-A ^d (B/c-specific)	
	Inflammatory cells	Vessel	Inflammatory cells	Vessel
Isograft (B6 to B6)	1.1 $\pm$ 0.2	0.1 $\pm$ 0.1	NA	NA
Allograft (B/c to B6); 7 days, with no treatment	3.6 $\pm$ 0.3	0.1 $\pm$ 0.1	1.6 $\pm$ 0.5	3.1 $\pm$ 0.2
Allograft (B/c to B6); 8 weeks, treated with MAbs	3.6 $\pm$ 0.3	0.3 $\pm$ 0.2	0.9 $\pm$ 0.5	2.7 $\pm$ 0.7
Allograft (B/c to B6); 12 weeks, treated with MAbs	2.2 $\pm$ 0.8	0.0 $\pm$ 0.0	0.5 $\pm$ 0.2	2.0 $\pm$ 0.9

Values represent the mean $\pm$ SD; grading is as in Table 2. NA, not applicable.

Figure 2. Histological appearances of acute parenchymal rejection in short-term allografts *versus* graft arterial disease in long-term allografts. **A:** A representative paraffin section of an allograft heart (B/c to B6) 7 days after transplantation. H&E; magnification, $\times 100$. Note the multifocal, severe infiltration of mononuclear inflammatory cells in the parenchyma and perivascular areas. **B:** A representative paraffin section of an allograft heart (B/c to B6) 12 weeks after transplantation (elastin stain; magnification, $\times 100$), showing marked fibrointimal expansion and complete luminal occlusion, morphologically resembling typical human graft arteriosclerosis.

perivascular infiltrates (Table 3; Figures 5 and 6). Twelve-week allografts in immunosuppressed recipients showed findings comparable to 8-week allografts but displayed overall lower levels of class II expression (Table 3; Figure 6). No myocyte staining for MHC II of either haplotype was ever seen, and in no case did allograft ECs or SMCs express recipient MHC class II molecules.

To demonstrate unambiguously that vascular wall ECs were of donor origin, double-label immunohistochemistry was performed on allografts 12 weeks after transplantation. In this protocol, ECs are stained blue and MHC II positivity imparts a red-brown color; double-stained cells have a deep purple cast. Samples stained for CD31 and with nonspecific control antibodies isotype matched to the MHC-II-specific antibodies (Figure 7A) show a rim of EC staining on the vascular lumen and only background staining of the vessel wall and surrounding interstitial tissue. Double staining for CD31 and recipient MHC II (I-A^b) shows blue luminal staining of ECs as well as strong I-A^b positivity in intramural and perivascular inflammatory cells, demonstrating the host origin of the inflammatory cells; ECs are not double stained (Figure 7B). In contrast, staining for CD31 and donor MHC II (I-A^d) shows strong double staining in the vessel lumen,

Figure 3. Immunohistochemical staining for cell type and MHC II expression in normal hearts and isografts. **A:** A freshly explanted, native B6 heart stained with anti I-A^b MAb, showing no constitutive MHC class II expression in any cell. Magnification, $\times 200$. **B:** B6 isograft, anti-CD4 and anti-CD8 MAbs given weekly, 12 weeks after transplantation; staining for Mac-3, a marker of activated macrophages. Sparse infiltrating macrophages are observed in epicardium and adjacent area (arrows). Magnification, $\times 50$. **C:** B6 isograft, anti-CD4 and anti-CD8 MAbs given weekly, 12 weeks after transplantation; staining for I-A^b. MHC class II expression in the isograft is largely confined to epicardial macrophages (arrows). Magnification, $\times 50$.

Allograft / 7 days

Figure 4. Immunostaining for cell type and MHC II expression in acutely rejecting allografts. Representative serial sections of a B/c allograft heart transplanted into a B6 recipient without immunosuppressive treatment, failing at 7 days after transplantation. Magnification, $\times 200$. **A:** Staining for CD45. There is a severe perivascular infiltration of mononuclear inflammatory cells. **B:** Staining for Mac-3 (representing activated macrophages), revealing that most of the perivascular inflammatory cells are Mac-3 positive. **C:** Staining for CD31 (PECAM-1), showing endothelium of the large arteries and the adjacent capillaries. **D:** Staining with 1A4, showing smooth muscle α -actin-positive cells in the media of the vessel. **E to H:** Immunofluorescent staining (Texas Red) for MHC class II molecules (**E** and **F**) and isotype-matched controls (**G** and **H**). Magnification, $\times 200$. **E:** Staining for I-A^b (recipient MHC class II). Inflammatory infiltrates around a vessel, but not vessel wall cells, express recipient MHC class II molecules. **F:** Staining for I-A^d (donor MHC class II). ECs and SMCs in the vessel express abundant donor MHC class II molecules. Occasional inflammatory cells around the vessel likely represent passenger leukocytes. The parenchymal staining of myocytes is due to background autofluorescence (see **G** and **H**). **G:** Control for **E**, using nonspecific biotin-conjugated mouse IgG_{2a} as the first antibody. **H:** Control for **F**, using nonspecific biotin-conjugated mouse IgG_{2b} as the first antibody.

Allograft / 8 weeks

Figure 5. Immunostaining for cell type and MHC II expression in allografts 8 weeks after transplantation. Representative serial sections are of B/c allograft hearts transplanted into B6 recipients immunosuppressed with anti-CD4 and anti-CD8 MABs. Magnification, $\times 200$. **A:** Staining for CD45, showing vascular leukocyte infiltration, especially involving the intima and perivascular area. **B:** Staining for Mac-3. An abundant macrophage infiltrate is present in the expanded intima of the vascular lesion and in the perivascular area. **C:** Staining for CD31 (PECAM-1), showing endothelium of the artery and the adjacent microvessels. Staining with 1A4, showing smooth muscle α -actin-positive cells in the media and the expanded intima of the vascular lesion. **E to H:** Immunofluorescent staining (Texas Red) for MHC class II molecules (**E** and **F**) and isotype-matched controls (**G** and **H**). **E:** Staining for I-A^b (recipient MHC class II). Inflammatory cells in the expanded intima, and around the vessel express recipient MHC class II molecules; ECs and medial SMCs do not express recipient MHC class II molecules. **F:** Staining for I-A^d (donor MHC class II). ECs and medial SMCs in the vessel express donor MHC class II molecules intensely, although SMCs in the expanded intima do not express any I-A^d molecules. The signal from parenchymal myocytes results from background autofluorescence (see **G** and **H**). **G:** Control for **E**, using nonspecific biotin-conjugated mouse IgG_{2a} as the first antibody. **H:** Control for **F**, using nonspecific biotin-conjugated mouse IgG_{2b} as the first antibody.

Allograft / 12 weeks

Figure 6. Immunostaining for cell type and MHC II expression in allografts 12 weeks after transplantation. Representative serial sections are of B/c allograft hearts transplanted into B6 recipients immunosuppressed with anti-CD4 and anti-CD8 MAbs. Magnification, $\times 200$. **A:** Staining for CD45, showing leukocyte infiltration in the intima and perivascular area. **B:** Staining for Mac-3, showing abundant macrophage infiltration in the expanded intima and perivascular area. **C:** Staining for CD31 (PECAM-1), showing the endothelial layer of the artery and adjacent smaller vessels. **D:** Staining with 1A4, showing smooth muscle α -actin-positive cells in the media and the expanded intima of the vascular lesion. **E to H:** Immunofluorescent staining (Texas Red) for MHC class II molecules (**E** and **F**) and isotype-matched controls (**G** and **H**). **E:** Staining for I-A^b (recipient MHC class II). Inflammatory cells in the expanded intima and around the vessel express recipient MHC class II molecules; ECs and medial SMCs do not express recipient MHC class II molecules. **F:** Staining for I-A^d (donor MHC class II). ECs and medial SMCs in the vessel express donor MHC class II molecules intensely, although SMCs in the expanded intima do not express I-A^d molecules. The signal from parenchymal myocytes results from background autofluorescence (see **G** and **H**). **G:** Control for **E**, using nonspecific biotin-conjugated mouse IgG_{2a} as the first antibody. **H:** Control for **F**, using nonspecific biotin-conjugated mouse IgG_{2b} as the first antibody.

Allograft/12 weeks

Figure 7. Double-staining immunohistochemistry for CD31 and MHC II molecules in allografts 12 weeks after transplantation. Representative sections of B/c allograft hearts transplanted into B6 recipients immunosuppressed with anti-CD4 and anti-CD8 MAbs. Magnification, $\times 100$. **A:** Double staining with anti-CD31 (blue stain) and nonspecific biotin-conjugated mouse IgG_{2b} (red-brown; control for anti-I-A^d); lightly counterstained with hematoxylin. Note staining of the endothelial layer of the artery (arrowheads) and the level of background red-brown staining of the vessel wall and surrounding interstitium. **B:** Double staining with anti-CD31 (blue stain) and anti-I-A^b (recipient MHC II; red-brown stain); lightly counterstained with hematoxylin. ECs are stained blue only, whereas inflammatory cells in the vessel wall and perivascular interstitium are strongly positive for recipient MHC II. **C:** Double staining with anti-CD31 (blue stain) and anti-I-A^d (donor MHC II; red-brown stain); lightly counterstained with hematoxylin. ECs are stained dark purple, reflecting double staining for both CD31 and donor MHC II. Smooth muscle cells of the vascular media and expanded intima are relatively weakly stained for MHC II.

demonstrating the donor origin of the ECs (Figure 7C); as in Figure 6, SMCs expressed relatively low levels of donor MHC II.

Discussion

A number of cytokines can regulate MHC class II expression by the various constituent cells in allografts; for example, MHC II on human ECs is induced by interferon ($\text{IFN-}\gamma$),¹³ and MHC II on murine macrophages may be increased by $\text{IFN-}\gamma$,¹⁴ tumor necrosis factor- α ,^{15,16} and/or interleukin-4.¹⁷ MHC class II expression on graft endothelium has particular importance as a major determinant of graft immunogenicity.^{2,13,18,19} Previous reports demonstrated that ECs in acutely rejecting allografts express donor-specific class II molecules.⁴⁻⁶ However, it was unclear whether donor or recipient ECs populated the engrafted vessels in solid organs transplanted for prolonged periods. This study used long-term (8- to 12-week) cardiac allografts in mice with MHC-mismatched donors and recipients to establish the origin of the cells that express class II molecules. Although MHC II expression is a critical component in the stimulation of CD4^+ T cells, it should also be emphasized that a number of co-stimulator molecules, beyond the scope of this study (eg, CD40, CD80, and CD86), likewise modulate the local immune response. We have focused on MHC II expression because it plays a central role in T cell stimulation by foreign tissues; persistent donor MHC II would therefore be important in the pathology of long-term allograft failure.

The immunosuppressive protocol used in these experiments permits a single, early 4-day episode of untreated rejection, followed by virtually complete CD4 and CD8 cell ablation and therefore long-term suppression of any further immune-specific response.⁹ The model permits long-term (at least 12 weeks) survival of complete allogeneic-mismatched allografts. Moreover, this immunosuppression protocol yields graft arteriosclerosis lesions histologically identical to those seen with conventional pretransplant MAb treatment.^{9,10} Although these long-term grafts lack CD4 and CD8 T lymphocytes, they nevertheless exhibit an extensive infiltrate of MHC-II-positive macrophages and levels of adhesion molecules (CD54 (ICAM-1) and CD106 (VCAM-1)) comparable to what we have described in allografts with other immunosuppression protocols.^{9,10} Furthermore, the absence of $\text{IFN-}\gamma$ abrogates graft arteriosclerosis lesions in this model with early transient acute rejection, in a manner analogous to what we have described using pretransplant immunosuppression.^{9,10,20} We therefore believe that the underlying pathogenic mechanisms of graft arteriosclerosis in this model do not substantively differ from those described by ourselves and others.^{9,10,20} In addition, the same persistence of donor MHC-II-positive ECs and SMCs is seen in complete allogeneic-mismatched allografts 12 weeks after transplantation, using rapamycin immunosuppression where T cell ablation does not occur (S. Hasegawa H. Nagano, P. Libby, and R. N. Mitchell, in preparation).

Normal nontransplanted mouse hearts in this study do not show any class II expression, although a previous study demonstrated the presence of class-II-positive dendritic cells in normal rat hearts.⁴ The class-II-positive cells in the rat heart study might reflect a low-level inflammatory stimulation due to pathogens. Our results support the contention that MHC class II expression in normal mouse heart tissue (ECs and SMCs) is inducible but not constitutive. Moreover, in agreement with others, we find no immunohistochemical evidence of MHC II expression on cardiac myocytes.²¹

In long-term isografts, only occasional macrophages in the epicardium express MHC class II molecules. In contrast, acutely rejecting allografts without treatment and allografts from long-term (8 to 12 weeks) immunosuppressed recipients both show intense expression of recipient-specific class II molecules in infiltrates around vessels but not in vascular ECs or SMCs. In the long-term allografts, mononuclear inflammatory cells in the expanded intima also express recipient-specific MHC class II molecules. The mononuclear cells expressing recipient MHC class II are likely macrophages,^{22,23} as they also stain for Mac-3; although B cells also bear MHC class II, they are generally sparsely represented in these allografts (not shown). Activated T cells in mice do not express class II molecules²⁴; moreover, the immunosuppressive regimen used in these experiments (weekly anti-CD4 and anti-CD8 MAbs) deletes the vast majority of T cells from the periphery as well as the allograft.⁹ The findings suggest that sustained expression of host MHC class II molecules on infiltrating macrophages (host antigen-presenting cells) could enhance immune responses even in long-term allografts.

The ECs and medial SMCs of acutely rejecting allografts express donor-specific MHC class II molecules, in accord with previous work.⁴⁻⁷ Although other studies have reported that graft ECs express donor-specific MHC class II molecules in the first two weeks after transplantation, our study demonstrates that, in allografts up to 12 weeks, ECs and SMCs express predominantly donor-specific MHC class II molecules. This finding suggests that ECs and SMCs in long-term allografts largely derive from the donor and that ongoing, allo-restricted (direct) antigen presentation by these vascular wall cells may contribute to a sustained local immune response during the development of graft arteriosclerosis. We did not examine the origin of vascular wall cells beyond 12 weeks; however, the absence of any discernible recipient ECs or SMCs, even after substantial vascular pathological change has occurred, demonstrates the ability of donor cells to persist for long periods of time.

Although the medial SMCs predominantly derive from donor cells, it is much more difficult to establish the origin of the SMCs of the intimal fibroproliferative region. Despite strong MHC II immunoreactivity of ECs and medial SMCs, the SMCs accumulating in the intima consistently showed very poor staining for MHC II of either donor or host haplotype. This result agrees with previous work demonstrating an inverse correlation between SMC proliferation and MHC II expression.²⁵ Accordingly, sites

with the greatest growth, such as the intima of graft arteriosclerotic lesions, would be expected to have the lowest surface expression of MHC II.

Plissonnier et al,²⁶ in a rat aortic allograft model, showed that the SMCs in late (2 months) intimal hyperplastic lesions may derive from the allograft recipient. However, in these aortic allografts, the donor EC and SMC populations die within the first 1 to 2 weeks after transplantation and would be unable to repopulate the graft. Because of the extensive early aortic wall necrosis, the later predominance of recipient cells is therefore not surprising. Moreover, these experiments did not determine how many of the intimal cells were in fact infiltrating inflammatory cells. In the cardiac allografts, in contrast, we do not see any substantial vascular wall necrosis, and we can identify which cell type is present in the intimal hyperplastic lesions. As shown in Figures 5 and 6, infiltrating recipient inflammatory cells can be readily demonstrated in the areas of intimal hyperplasia. Likewise, we can demonstrate the presence of smooth muscle α -actin-positive cells in these areas but cannot establish their MHC II haplotype. Consequently, the origin of the proliferating intimal SMCs is uncertain. However, given that the allograft vascular media is derived from the donor, it seems most likely that the bulk of the intimal SMCs would also be largely donor derived.

Occasional cells around vessels in acutely rejecting allografts express donor-specific MHC class II molecules, most likely representing passenger leukocytes.^{27,28} A previous study in human allografts showed that dendritic cells were present in the vascular lesions.²⁹ In our study, however, the class-II-expressing donor antigen-presenting cells decrease with time and have mostly disappeared by 12 weeks after transplantation. The role of passenger leukocytes in the sustained regional immune response in coronary arteries therefore appears limited, but it is conceivable that MHC class II expression by passenger leukocytes or donor dendritic cells might be implicated in long-term allograft acceptance. Tolerance induction by the mechanism of microchimerism has been suggested to reduce the development of graft arteriosclerosis.^{30,31}

In conclusion, we have demonstrated in long-term cardiac allografts that infiltrating inflammatory cells largely express recipient-specific MHC class II molecules and that graft ECs and medial SMCs express donor-specific MHC class II molecules. These results suggest that donor-derived antigenic peptides binding to MHC molecules on host macrophages can contribute to indirect CD4⁺ T cell activation in long-term allografts. Concurrently, persistent allogeneic donor MHC class II molecules on graft ECs and SMCs can also contribute to a sustained direct activation of host CD4⁺ T cells. It is not possible to specifically quantitate the relative contribution of host *versus* donor MHC II to the immunological response; it is nevertheless consequential that numerous donor ECs and SMCs, capable of expressing foreign MHC antigens, persist long into the life of a solid-organ allograft.

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